Peer review of cyanotoxin toxicity criteria and health based water concentrations to protect human swimmers, dogs and cattle

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1. **General Approach and General Comments of the Reviewer**:
    a. The OEHHA report addresses the need for informing the public of the potential public and animal health threats of cyanotoxins. As with any rapidly emerging environmental problem, the scientific data are often complex and, in some cases, contradictory.
    b. The six candidate cyanotoxins selected seem reasonable and appropriate considering the 1) widespread occurrence the toxins 2) high toxicities related to humans and wildlife and 3) scientific literature available on these cyanotoxins at this time. The OEHHA has reviewed and discussed the relevant literature on the six candidate cyanotoxins, up to the date of release of the draft proposal. Although the toxicology of cyanobacteria is a rapidly developing field with a growing literature, this reviewer is not aware of any findings published since 2009 that would significantly alter the findings of the OEHHA report.
    c. The report does not deal with the question of analytic methods needed to quantify the six cyanotoxins, an important but perhaps not critical to the immediate goals of the report. This point is covered later in more detail.

2. **Toxicity Criteria for the six chemicals**

    a. Epidemiological studies in China suggest possible long-term effects of microcystins on the incidence of liver cancers (Falconer 2005, Yu et al. 2002, Yu & Yuan 2004). However, as noted by the authors, such studies cannot exclude the role of other toxic substances as well as other microbes associated with polluted waters. In the absence of controlled long-term experiments on carcinogenic effects of microcystins, the focus of the OEHHA report on liver toxicity is well justified.

    b. The OEHHA use of studies using cyanobacteria extracts to set toxin action levels raises some important considerations. On the one hand, experiments using a single purified toxin allow for clear association

between the toxin level and the response. In contrast, extracts of cyanobacteria contain a measurable concentration of the toxin of interest, but cyanobacteria extracts often contain additional toxic substances as well as a broad spectrum of other chemicals, with unknown effects on the test species. Thus, one might assume that tests conducted with purified toxins would be the ideal basis for setting toxicity criteria. However, an additional factor should be considered, i.e. the cyanotoxins under consideration are generally endotoxins, contained within the cyanobacteria cells, unless released through decomposition or cell breakage, such as through sonification and freeze-thaw treatments commonly employed in preparation of samples for testing. Thus, for example, organisms consuming cyanobacteria contaminated water would ingest a mixture of both dissolved and intracellular toxin. The free dissolved fraction might be expected to correspond to the effects seen with the purified toxin, whereas the toxin contained in the cells, often a large portion of the toxin present, is not available to become toxic until it is released in the digestive system. There is little known about the ability of humans or other mammals to digest cyanobacteria, although it is likely to be variable, depending on the type and condition of cyanobacteria cells. Based on studies of with crustacean zooplankton such as *Daphnia*, cyanobacteria with protective gelatinous sheaths may pass through the gut unharmed (Porter 1975). Thus, studies employing purified cyanotoxins might be expected to overestimate the effect of the single toxin, but do not allow for the combined or synergistic effects caused by other chemical and cyanobacteria metabolites present and thus may underestimate the toxicity that would occur in nature. Considering the paucity of studies with vertebrates using purified toxins, especially cylindrospermosin and anatoxin-a, the OEHHA decision to use the results from studies using cyanobacteria extracts seems reasonable as the best information available at this time.

c. ***Technical points***:

i. In Table 13, it is not clear how the anatoxin-a subchronic level of 100 for dairy was derived and whether it is based on experimental data or assumptions. This section is somewhat confusing and difficult to follow.

ii. Table 4 footnote #3 states that "apply action levels to the sum of all microcystins variants until subchronic toxicities of other variants are clarified". There is ambiguity as to whether "all microcystins variants" refers to the four microcystins variants considered in this document or the broader array of microcystins analogs that would be measured with an ELISA technique.

iii. Laboratory techniques used for analysis of cyanotoxins in tissues may involve the use of fresh tissue or freeze-dried material. To avoid confusion and potential errors, where tissue levels are concerned it is important to clearly state whether the units for the tissue are wet weight or dry weight (e.g. Table 4. This could be done as a footnote or as often is done in the units of measure as ng/g tissue dw or ww.
iv. Concerning the table on page, footnote 4 should probably read "subchronic" rather than "subacute".

3. Exposure Assessment

a. ***MC in aerosols***:

Recent studies conducted along coastal beaches have confirmed the presence of red tide neurotoxins in ocean aerosols generated by bubbles. These studies also confirm a correlation between the concentration of the toxin in the water and in the aerosols. Less is known about the importance of aerosol-borne cyanotoxins on or near lakes.

Numerous reports suggest a higher incidence of illnesses such as flu-like symptoms, rashes and respiratory irritation in persons living near lakes with cyanobacteria blooms (Stewart et al. 2006), although there are few quantitative data to support these claims. Intake of cyanotoxins in airborne aerosols represents a potentially important pathway, because of the broad range of dispersal potential exposure to large populations. The few studies that have been conducted on aerosols emitted from water bodies have found microcystins present, but in low concentrations. The OEHHA dismisses aerosols as a potential source of microcystins for lake recreation users based on the data from Cheng (2007) that had some field results, but focused largely on laboratory examination of aerosol formation. More relevant data were collected in a recent study by Backer et al. (2010) of two California lakes that found an average of 0.3 ng MC/m3 (<0.1 – 2.89 ng MC/m3) in the aerosols collected at these lakes during periods of cyanobacteria blooms. Assuming an adult inhalation rate of 25 liters per minute, Backer et al. estimated an inhalation of 0.8 ng MC for a 106 min exposure of swimming or boating. It also appeared from this study that most of the inhaled toxins were deposited on the upper respiratory tract, a potentially effective area for absorption of the toxins into the body. These calculations were based on average recreational periods of less than two hours, however, and do not consider that in addition to the period of active recreation many lake visitors are likely to spend a significant amount of time at or near the water body at other activities, thereby having considerably longer

exposure times. Of course, exposures would be even longer for residents living at or working near the lake.
Although this newer information on toxins in aerosols near lakes suggests inhalation could be a pathway for exposure to cyanobacteria toxins, it would appear that OEHHA action level for microcystins in lake water of 0.7 μg MC/L should provide an adequate level of protection against inhalation of harmful levels of MC in the air during recreation activities. Further studies are badly needed to evaluate long-distance dispersal and potential long-term effects of aerosolized hepatotoxins and neurotoxins on both lake users and lake residents.

b. ***Exposure of dogs to cyanotoxins*:**

Estimation of action levels of microcystins, anatoxin-a and cylindrospermopsin for dogs is especially difficult to determine. The OEHHA has accounted for the intake by dogs of water and cyanobacteria attached to the fur following swimming. The assumed ingestion of a 2 mm coating of water seems plausible, although this is likely to be variable and dependent on the size and breed of the dog. It is perhaps more difficult to account for the highly selective drinking habits of dogs, that generally drink close to the shore, where concentrations of cyanobacteria also tend to be the highest, especially when surface blooms are blown shoreward. Thus, dogs may be exposed to higher toxin levels than those sampled at sampling stations somewhat offshore. Also problematic is the possibility that some animals, including dogs, may be attracted to water containing cyanobacteria (Codd et al. 1992, Lopez et al. 1999) and thus may actively select to drink from areas of the lake with highest cyanobacteria concentrations and the highest levels toxicity. According to the above authors, this "fatal attraction" may be responsible for the frequent reports of acute deaths of dogs and cattle after exposure to lakes and streams. Selective near shore drinking is potentially a more important consideration that the gulping of water during swimming.

The acute action canine drinking water exposure levels of 500, 400 and 500 μg/L for microcystins, anatoxin-a and cylindrospermopsin, respectively, would seem adequate, but dependent on a sampling protocol that actually collects water for testing from the immediate shoreline area that dogs would normally use for drinking. Based on the limited studies available, the subchronic action levels recommended are likely to provide safe levels of cyanotoxins where repeated exposures are expected. It is important to consider that action levels are only meaningful when tests are conducted on the near shore water that is likely to be consumed by dogs. Although it

would be difficult to develop an accurate metric to account for selective drinking, it might be useful to consider the addition of an uncertainty factor to account for the tendency for near shore drinking and the possible attraction to higher than average levels of cyanotoxins.

c. ***Cyanobacteria crusts:***
Presumably, many of the crusts of cyanobacteria deposited on the shores of ponds, lakes and streams are the result of surface cyanobacteria blooms of planktonic cyanobacteria that have aggregated along the shore or blown on land and. Benthic cyanobacteria often form dense mats that periodically rise to the surface, buoyed by gas that has accumulated underneath the mat. It seems probable that some of the crusts that occur on shore could be derrived from floating benthic mats. There is evidence that attached forms of benthic cyanobacteria such as *Phormidium* and *Oscillatoria* produce microcystins and anatoxin-a and ingestion of dislodged mats have been linked to the death of cattle in Switzerland (Mez et al. 1997) and dogs in Scottland and France (Gugger et al. 2005, Edwards et al. 1992). Thus, OEHHA might consider including benthic mats in the category with "crusts", since it is likely that at times these are one and the same. This designation might also result in an awareness of the potential risks associated with submerged, floating and landed cyanobacteria mats and the determination of their toxicity.

4. ***Microcystin Ecotoxicology***:

The OEHHA review of the research on ecotoxicology of cyanotoxins included an reasonable sampling of papers published in this field as well as an accurate assessment of the state of the understanding for the microcystins, cylindrospermosin and anatoxin-a. As noted in the OEHHA report, most of the research on aquatic food webs has examined the production and accumulation of microcystins in components of the web, including zooplankton, fish and, to a lesser extent, freshwater mussels. Although microcystins do not generally biomagnify as has been seen for some toxins, such as DDT and mercury, trophic levels do retain the relatively stable microcystins so that they are effectively transferred to the higher trophic levels (Kotak et al. 1996).

Since most studies conducted on cyanotoxins in lake food webs have measured static quantities of toxins in the various trophic levels we have little understanding about the dynamics of the transfer of cyanotoxins in lake ecosystem. For example, bloom forming cyanobacteria such as *Anabaena* and *Microcystis* are relatively large forms and are highly inedible for many of the zooplankton grazers,

such as *Daphnia*. Thus, the seemingly simple question of how toxins enter and move through the food web cannot be answered at this time. Also, it is not known whether some of the toxicity detected in lake water is actually produced by the smallest cyanobacteria or picocyanobacteria (< 2 μm). These abundant phytoplankton are not considered in most studies of cyanobacteria toxicity, although these potentially grazeable cells are capable of producing microcystins (Domingos et al. 1999).

The OEHHA accurately concluded that the research to date is inadequate to allow for setting toxin limits to protect fish species. Among the many toxicological issues that are at present inadequately addressed are 1) differences in the tolerance levels of fish species to the biotoxins 2) ontogenetic changes in sensitivity to the toxins with age of the fish and 3) the ability of some fish and invertebrates (Williams et al. 1997, Smith et al. 2010) to covalently bind toxins such as microcystins to proteins where they are effectively stored in a non-toxic state and possibly slowly released through excretion (Smith and Haney 2006).

5. **Broader perspective points and questions**:
    a. ***Data availability***: The report entitled "Toxicological summary and suggested action levels to reduce potential adverse health effects of six cyanotoxins" is comprehensive and clearly describes the rationale and scientific basis for the toxicity and exposure assessments as well as the proposed action levels for the six cyanobacteria toxins under consideration. The subject is complex and many areas in this field have had little research, such as the carcinogenic potential of cyanobacteria toxins. Also, there is little known about the potential effects of chronic exposure to the neurotoxins, such as anatoxin-a.

    b. The OEHH report makes an important and necessary step in updating the recommendations of the World Health Organization, still widely used although it was first proposed in 1998. The proposed action levels for human recreation, dogs and cattle make useful distinctions between target types (humans, dogs and cattle) as well as between acute and subchronic effects, where possible. It is not clear how these categories will be eventually applied to specific situations, although it appears this information is designed to assist state and local agencies in setting appropriate limits.

    c. ***Testing methods***: Although methodologies for measuring the candidate cyanotoxins was not in the OEHHA report, implementation of these findings will require a review and careful analysis of the most appropriate detection methods. Development of SOPs for the testing

of cyanotoxins will not be simple. For example, the report clearly identifies the four microcystins analogs, LR, YR, RR and LA, selected in in part because they had comparable RfD levels. To determine the concentrations of each analog at this time one could use HPLC-MS. From a practical standpoint, however, state and local agencies may find it more efficient and less costly to measure the microcystins levels with an ELISA kit, as this is highly sensitive, can be carried out with minimal laboratory facilities and personnel. The results of the testing, however, will differ with the two methods, as ELISA antibody reactions generally have a wide range of cross reactivity, measuring more than the four selected MC analogs, and doing so with differing degrees of reactivity. Considering the importance of turn-around-time for getting samples tested when public health is involved, the ELISA method may be preferable, but it will not be possible to know which microcystins were present if that technique is used.

Bound and free forms of MC: Microcystins are generally extracted by exposure of tissues to aqueous methanol. This treatment does not extract microcystins that are covalently bound to proteins. Williams et al. (1997) raised the question of the importance of microcystins bound in cells to protein phosphatases when they determined that the majority of the total body load of MC in blue mussels and Dungeness crab was present in the protein bound form. This and other studies using Lemieux oxidation to release the bound MC have indicated that a large fraction of the total MC pool in organisms is in the covalently bound form. However, the relevance of this finding is not clear, since the covalently bound MC is presumably non-toxic to the organism containing it, although and there is evidence that bound MC may contribute to the transfer of MC through the food web (Smith et al. 2010).

d. ***Surrogate methods***: It is difficult to ignore the difficulties and costs associated with measuring cyanobacteria toxins. As noted in the OEHHA report, many states have employed testing procedures that utilize counts of cyanobacteria cells as a proxy for toxicity testing. Although the OEHHA report deals solely with cyanobacteria toxins, it might be useful to examine other methods as surrogates for estimating the risk from toxic cyanobacteria. Despite many limitations, one of these methods that shows promise is the use of fluoroprobes that measure the fluorescence of phycobilin pigments found in cyanobacteria. When calibrated with standardized laboratory culture of a known strain of cyanobacteria such as *Microcystis aeroginosa*, rapid assessment can be made of the total population of cyanobacteria, Leboulanger et al (2002) has demonstrated phycocyanin fluorescence counts can be used to predict the levels of cyanobacteria and the probable levels of microcystins

present. This relationship works best at high concentrations of cyanobacteria, when potentially interfering forms such as cryptophytes are not abundant (McQuaid et al. 2011). The advantages of this method, when properly calibrated, are that it is rapid, relatively inexpensive and the measurements can be conducted either at the *in situ* at the lake. Rapid assessment can be especially important when evaluating water condition in recreational waters, since the local conditions in a particular region of the lake can change rapidly, depending on weather conditions. As with any method there are potential problems that must be addressed including: 1) microscopic examination should also be conducted to assure that other phycocyanin/phycoerythrin containing phytoplankton such as cryptomonads and dinoflagellates are not present 2) background fluorescence by colored dissolved organic matter may create errors in humic rich waters and 3) quenching of fluorescence signals at high turbidity levels may result in underestimates of cyanobacteria abundance. Because of the need for a rapid cyanobacteria assessment method, major producers of these fluorescence probes are currently developing methods that will improve the accuracy of this technique, such as built-in corrections for high turbidity and humic water color. As these methods are further tested and improved they may prove to be a valuable addition to the direct measurement of cyanotoxins. For example, on-lake fluorometry could provide the first level of indication of a water quality problem that could be followed up with more accurate analysis of the cyanotoxins present.

e. ***Sampling protocols***: Bloom-forming cyanobacteria present a particularly challenging sampling problem, since their buoyancy and relatively large size give them in-lake mobility not generally seen with other toxins found in lakes. The most sophisticated water testing procedures mean little if the sampling is not carefully conducted. It would be especially useful if the OEHHA could develop recommended sampling protocols for the different water bodies likely to be involved in cyanobacteria testing. The objectives of the sampling must first be determined, e.g., is the intent of the sample to provide evidence of the average conditions for the water body, or to represent the condition of an isolated region such as a beach or recreation area. The sampling must also consider the vertical mobility of cyanobacteria blooms, especially if these are concentrated at the water surface. Since many of the commonly occurring cyanobacteria such as *Anabaena*, *Planktothrix* and *Microcystis* can adjust their buoyancy according to light conditions, coming to the surface under low light or at night and easily mixing deeper in the water column with bright light and with light wind action. Thus, for example, it is important to avoid using grab samples that may hit or miss the population, depending on the depth sampled. Collection with a form of integrated tube sampler

would minimize spatial variability due to depth. The horizontal distribution across the lake is also transient and highly patchy, requiring an integrated horizontal sampler (our lab uses a continuous peristaltic pump) or multiple sampling sites. Sampling designs may follow a general protocol, but specifics will most likely vary with each system, dependent on the lake or pond size and morphometry. I emphasize this last point, as it is not often considered as a fundamental part of water quality testing programs, despite its potential importance.

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